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Pharmacological Characterization of Bosentan, a New Potent Orally Active Nonpeptide Endothelin Receptor Antagonist

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ABSTRACT

The authors describe here the pharmacological properties of bosentan, a new nonpeptide mixed antagonist of endothelin (ET) receptors, obtained by structural optimization of the less potent Ro 46-2005 [Ro 46-2005 (4-tert-butyl-*N*-[6-(2-hydroxyethoxy)-5-(3-methoxy-phenoxy)-4-pyrimidinyl]-benzenesulfonamide]. Bosentan (Ro 47-0203, 4-*tert*-butyl-N-[6-(2-hydroxy-ethoxy)-5-(2-methoxy-phenoxy)-2,2′-bipyrimidin-4-yl]-benzenesulfonamide) competitively antagonized the specific binding of [^{125}I]-labeled ET-1 on human smooth muscle cells (ET_A receptors) with a K_i of 4.7 nM and on human placenta (ET_B receptors) with a K_i of 95 nM. It also inhibited the binding of selective ET_B ligands on porcine trachea. Contractions induced by ET-1 in isolated rat aorta (ET_A) and by the selective ET_B agonist sarafotoxin S6C in rat trachea were competitively inhibited by bosentan (pA_2 = 7.2 and 6.0, respectively), as was the endothelium-dependent relaxation to sarafotoxin S6C in rabbit superior mesenteric artery (pA_2 = 6.7). The binding of 40 other peptides, prostaglandins, ions and neurotransmitters was not significantly affected by bosentan, which shows its specificity for ET receptors. *In vivo*, bosentan inhibited the pressor response to big ET-1 both aft r i.v. and oral administration, with a long duration of action and no intrinsic agonist activity. It also inhibited the depressor and pressor effect of ET-1 and sarafotoxin S6C. Thus, bosentan is the most potent orally active antagonist of ET receptors described so far. Its pharmacological profile makes bos ntan a potentially useful drug in the management of clinical disorders associated with vasoconstriction.

ET-1, besides its potent and prolonged vasoconstrictor effect, may exert various biological actions in rodents and other mammals. It plays a role in the regulation of water and electrolyte balance, in the modulation of secretion of a number of hormones, in cell proliferation and in vascular permeability (Miller *et al.*, 1993; Rubanyi, 1992).

So far, three ET receptor subtypes have been described. Arai *et al.* (1990) and Sakurai *et al.* (1990) reported the cloning of complementary DNAs encoding, respectively, an ET_A receptor that shows a high affinity for ET-1 and an ET_B receptor that has an equal affinity for ET-1 and ET-3. The ET_A receptor plays a predominant role in the vasoconstrictor effect of ET-1; ET_B receptor, which is present on endothelial cells, mediates endothelium-dependent relaxation (Randall *et al.*, 1989; Takayanagi *et al.*, 1991). Recently, an ET_B-like receptor, which is present on certain smooth muscle cells and mediates vasoconstriction, was described, based on *in vitro* (Harrison *et al.*, 1992; Moreland *et al.*, 1992; Shetty *et al.*, 1993; Gray *et al.*, 1993) and *in vivo* (Clozel *et al.*, 1992; Teerlink *et al.*, 1994; Williams *et al.*, 1991) pharmacological evidence. Both forms of ET_B receptors are activated by ET_B selective agonists, such as sarafotoxin S6C (Williams *et al.*, 1991) or BQ-3020 [BQ-3020 (N-acetyl-Leu-Met-Asp-Lys-Glu-Ala-Val-Tyr-Phe-Ala-His-Leu-Asp-Ile-Ile-Trp)] (Kobayashi *et al.*, 1993). The effects that result from their activation are insensitive to ET_A selective-antagonists, such as BQ-123 cyclo[D-Trp-D-Asp-Pro-D-Val-Leu] (Fukuroda *et al.*, 1992; Ihara *et al.*, 1992). For reasons of clarity, we tentatively use throughout this article the terms ET_{B1} and ET_{B2} receptors (Warner *et al.*, 1993) to describe the ET_B receptor that mediates endothelium-dependent relaxation and vasoconstriction, respectively. Functional data suggest subtype selectivity for certain ligands, such as IRL 1038 [Cys11-Cys15]-endothelin-1(11–21), which can inhibit the effects of ET_{B1}, but not ET_{B2}, receptor stimulation (Sudjarwo *et al.*, 1993). The sequence of the two ET_B receptors may differ because *in situ* hybridization analysis of rat ET_B messenger RNA showed strong signals in endothelial and epithelial cells and brain regions but did not reveal any significant expression of ET_B receptor in vascular smooth muscle cells (Hori *et al.*, 1992; Sakurai *et al.*, 1990). However, two studies that used human

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ABBREVIATIONS: CHO cells, Chinese hamster ovary cells; ET, endothelin; Sf9, *Spodoptera frugiperda* cells; pA_2, negative logarithm of the molar concentration of antagonist that causes a 2-fold parallel shift to the right of the agonist concentration-response curve.

tissues found the presence of a messenger RNA that encoded ET_B receptor in vascular smooth muscle cells (Davenport *et al.*, 1993; Winkles *et al.*, 1993).

ET-1 might play a role in chronic and acute diseases that are associated with focal or systemic vasoconstriction and perhaps also cellular proliferation, bronchoconstriction or inflammation (Miller *et al.*, 1993). The development of selective ET antagonists is essential to understand the pathophysiological roles of ET and could lead to new therapeutic approaches. We recently described the first orally active ET receptor antagonist, Ro 46–2005 (Clozel *et al.*, 1993a; Breu *et al.*, 1993). In this study, we report the pharmacological properties of bosentan, also called Ro 47–0203 or 4-*tert*-butyl-N-[6-(2-hydroxy-ethoxy)-5-(2-methoxy-phenoxy)-2,2′-bipyrimidin-4-yl]-benzenesulfonamide (fig. 1), which was obtained by structural optimization of Ro 46–2005. We describe the *in vitro* and *in vivo* effects of bosentan and its capacity to inhibit the biological consequences of stimulation of the three receptors: ET_A, ET_{B1} and ET_{B2}. For this purpose, we developed specific test systems that allowed us to evaluate each one of the receptors separately. Overall, the results show that bosentan blocks all three ET_A, ET_{B1} and ET_{B2} receptors, is extremely specific for ET and is orally active.

Methods

Cell Culture

Baculovirus-infected insect cells (Sf9) were grown in 23-liter airlift fermentors, as described by Maiorella *et al.* (1988). Briefly, Sf9 cells were incubated at an initial cell density of 2.5×10^5 cells/ml by using IPL-41 medium (GIBCO Laboratories, Paisley, Scotland), which was supplemented with lipids and 1.5% fetal calf serum. The cells were grown for 4 to 5 days to a titer of 2.5 to 3.5×10^6 cells/ml and subsequently infected with recombinant baculovirus at a multiplicity of infection of 1. Three days after the infection, the cells were harvested by centrifugation (15 min, $800 \times g$) and frozen at −80°C.

Human vascular smooth muscle cells were obtained from umbilical veins and cultured as described (Gimbrone *et al.*, 1975). Briefly, after a first incubation with collagenase to remove endothelial cells, the umbilical vein was filled again with collagenase for 45 min. Smooth muscle cells were then grown to confluence in plastic flasks or 24-well plates in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum. They were cultured at 37°C in air that contained 5% CO_2. Smooth muscle cells were subcultured after trypsinization and studied between passages 4 and 6.

Rat mesangial cells were obtained, as described previously (Pfeilschifter *et al.*, 1984). They were grown in RPMI 1640 supplemented with 5 μg/ml of insulin, 5 μg/ml of selenious acid, 428 μg/ml of linoleic acid, 1 mg/ml of bovine serum albumin (ITS+, Collaborative Research, Bedford, MA), 10% fetal calf serum, 100 U/ml of penicillin, 100 μg/ml of streptomycin, 2.5 μg/ml of amphotericin B and 10 mM HEPES. They were studied at passages 10 to 20.

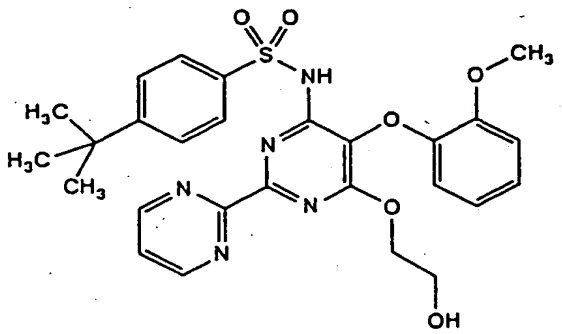


Fig. 1. Structure of bosentan.

CHO cells that expressed recombinant ET_A or recombinant ET_B receptor and were cloned from human placenta (Adachi *et al.*, 1991; Takasuka *et al.*, 1992) were grown in Minimal Essential Alpha Medium (GIBCO Laboratories, Paisley, Scotland) supplemented with 0.1 μM methotrexate, 5% dialyzed fetal calf serum, 100 U/ml of penicillin and 100 μg/ml of streptomycin.

Preparation of Membranes

Baculovirus-infected insect cells that expressed either recombinant ET_A or recombinant ET_B receptor and were cloned from human placenta were broken by three freeze-thaw cycles in hypotonic Tris buffer (5 mM, pH 7.4, 1 mM $MgCl_2$), resuspended in the same buffer with 250 mM sucrose and stored in aliquots at −80°C.

Microsomal membranes from human placenta, porcine cerebellum and porcine trachea were prepared as described earlier (Fischli *et al.*, 1989). Briefly, the tissues were homogenized in 5 mM Tris buffer, pH 7.4, which contained 1 mM $MgCl_2$ and 250 mM sucrose with a Polytron (Kinematica, Littau-Luzern, Switzerland) and subsequently with a Potter homogenizer (Vetter, Bender U. Hobeim, Zürich, Switzerland). After centrifugation at $3000 \times g$ for 15 min at 4°C, the supernatant was centrifuged again at $72{,}000 \times g$ for 40 min. The resulting pellet was finally suspended in 2.5 ml of 75 mM Tris buffer (pH 7.4), which contained 25 mM $MgCl_2$ and 250 mM sucrose, and was stored frozen at −80°C. Protein content was determined according to the method of Lowry *et al.* (1951) by using bovine serum albumin as a standard.

Binding Assays on Attached Cells

Binding experiments were performed as already described (Clozel *et al.*, 1989). Briefly, the cells (~10^5 cells per 16-mm diameter well) were washed three times and incubated at room temperature with 500 μl of binding medium (Dulbecco's modified Eagle's medium supplemented with 2 mg/ml of bovine serum albumin and 25 mM HEPES, pH 7.4), which contained [^{125}I]-labeled ET-1 (~60,000 counts/min; final concentration, 36 pM) and various concentrations of bosentan. After 2 hr, the cells were extensively washed with binding medium and solubilized in 1% sodium dodecyl sulfate with 0.5 M sodium hydroxide and 0.01 M EDTA at 37°C. The radioactivity of bound [^{125}I]-labeled ET-1 was measured (total binding). Nonspecific binding was determined simultaneously in the presence of 100 nM unlabeled ET-1. The maximal specific binding was calculated as the total binding minus the nonspecific binding. Specific binding represented 80% to 90% of total binding.

Binding Assays on Membranes

Suspensions of microsomal membranes were defrosted and centrifuged at $25{,}000 \times g$ for 10 min. The pellet was resuspended at 22°C in 50 mM Tris buffer (pH 7.4, 25 mM $MnCl_2$, 1 mM EDTA, 0.5% w/v bovine serum albumin). We used 50 μl of this suspension, which contained 5 μg (recombinant ET_A), 1 μg (recombinant ET_{B1}), 35 μg (placenta), 35 μg (porcine cerebellum) or 30 μg of protein (porcine trachea), in a 250-μl assay that contained the same buffer with 32 pM [^{125}I]-labeled tracer (ET-1, BQ-3020 or sarafotoxin S6C) and increasing amounts of unlabeled bosentan. After the 2-hr incubation at 22°C, bound and free ligands were separated by filtration. Each assay was performed three times in triplicate and nonspecific binding was assessed in the presence of 100 nM unlabeled ET-1. The K_i values were calculated with a direct-fit analysis of the binding curves using the LIGAND program (Munson and Rodbard, 1980). The Hill coefficients were taken as the slope of plots of log [percent bound/(100 − percent bound)] *versus* log concentration of bosentan.

Saturation Binding

Microsomal membranes of human placenta (35 μg of protein/well) and membranes of baculovirus-infected insect cells that expressed ET_A receptors (5 μg of protein/well) were used to perform saturation binding experiments with increasing amounts (6.4 to 660 pM) of [^{125}I]-labeled

ET-1 in the absence or in the presence of different concentrations of bosentan. Assay conditions were the same as those described for competition binding. The binding experiments were performed in triplicate and nonspecific binding was assessed in the presence of 100 nM unlabeled ET-1. K_D and B_{max} values were calculated by linear-regression analysis of Scatchard plots.

Selectivity Assay

The specificity of bosentan as an ET receptor antagonist was assessed by testing any possible interference with the binding of other regulatory agents. The binding of neurotransmitters (adenosine, catecholamines, acetylcholine, γ-amino-*n*-butyric acid, histamine, serotonin and glutamate), neuropeptides or intestinal peptides or growth factors (platelet-derived growth factor, transforming growth factor-β, angiotensin, arginine-vasopressin, bombesin, bradykinin, cholecystokinin, calcitonin gene-related peptide, substance P, neurokinin A, neuropeptide Y, neurotensin, somatostatin, thyrotropin-releasing hormone, vasoactive intestinal peptide, oxytocin and platelet activating factor), eicosanoids (leukotriene B_4 and thromboxane A_2) and ions (chloride, potassium and calcium) was tested using radiolabeled ligands and corresponding receptor preparations (Novascreen, Baltimore, MD).

In Vitro Functional Inhibitory Potency

Isolated rat aortic rings. Male 14- to 16-week-old Wistar-Kyoto rats were anesthetized with sodium thiobutabarbital (100 mg/kg i.p.) and the thoracic aorta was removed and cut into 5-mm rings. The endothelium was removed by gentle rubbing of the intimal surface and each ring was suspended in a 10-ml isolated organ chamber that contained gassed (95% O_2 and 5% CO_2) and warmed (37°C) Krebs-Henseleit solution of the following composition (in millimolar concentrations): NaCl, 115; KCl, 4.7; $MgSO_4$, 1.2; $KHPO_4$, 1.5; $NaHCO_3$, 25; $CaCl_2$, 2.5; and glucose, 10. The isometric force was recorded. The rings were stretched to a resting force of 3 g. After a 60-min equilibration period, the rings were contracted by using norepinephrine (10^{-7} M). Endothelium denudation was assessed by the absence of relaxation to acetylcholine (10^{-6} M). The rings were then washed and stretched if necessary until a stable base-line force was obtained. They were incubated with various concentrations (3×10^{-7} to 3×10^{-6} M) of bosentan. After 10 min, cumulative doses of ET-1 were added and the interval between doses was determined by the time required for the force generated to reach a plateau.

In another set of experiments, the rings were incubated with ET-1 for 60 min before the addition of various concentrations of bosentan. The relaxation of the preconstricted ring by bosentan was followed until a stable force was achieved. Each ring was subjected to only one concentration of bosentan.

Isolated rabbit superior mesenteric arterial rings. Superior mesenteric arteries were removed from male New Zealand White rabbits (3–4 kg, Biological Research Laboratories, Füllinsdorf, Switzerland) killed by an overdose of sodium pentobarbital. The arteries were cut into 4-mm rings and suspended in 10-ml organ chambers that contained warmed and gassed Krebs-Henseleit solution, as described earlier. The rings were stretched to a resting force of 2 g. After a 60-min equilibration period, the rings were contracted using norepinephrine (10^{-7} M). The presence of endothelium was verified by relaxation on the addition of acetylcholine (10^{-6} M). The rings were then washed and stretched if necessary until a stable base-line force was obtained. After a 10-min incubation with various concentrations of bosentan (10^{-7} to 10^{-5} M), the rings were contracted using either prostaglandin $F_{2\alpha}$ (10^{-7} M) or methoxamine (10^{-6} M), which in this preparation, gave more stable contractions than did norepinephrine. When a plateau of contraction was obtained, sarafotoxin S6C (10^{-11} to 10^{-7} M) was added cumulatively, the interval between doses was determined by the time required for the relaxation to reach a plateau. At the end of the experiment, acetylcholine (10^{-6} M) was added to ensure that maximal relaxation could be achieved. No quantitative difference was seen between rings contracted using prostaglandin $F_{2\alpha}$ and those contracted using methoxamine.

Isolated rat tracheal rings. Male 14- to 16-week-old Wistar-Kyoto rats were anesthetized with sodium thiobutabarbital (100 mg/kg i.p.) and the trachea was removed and cut into 5-mm rings. The epithelium was removed by gentle rubbing of the luminal surface and each ring was suspended in a 10-ml isolated organ chamber that contained gassed and warmed Krebs-Henseleit solution, as described earlier. The rings were stretched to a resting force of 2 g. After a 60-min equilibration period, the rings were contracted using potassium chloride (50 mM). The rings were then washed and stretched if necessary until a stable base-line force was obtained. After a 10-min incubation with bosentan (10^{-6} to 10^{-5} M), cumulative doses of sarafotoxin S6C were added. The interval between doses was determined by the time required for the force generated to reach a plateau.

Analysis and calculations. The maximum force was defined as the force generated with the highest concentration that yielded a maximal effect. From this, the ET-1 or sarafotoxin S6C concentration that yielded a half-maximal effect (EC_{50}) was calculated. Contractile and relaxant responses are expressed as a percentage of the maximal response. The pA_2 value, as an index of potency, was determined for each individual curve by the equation $pA_2 = \log(\text{concentration ratio} - 1) - \log[B]$, where concentration ratio is the ratio of EC_{50}values with and without the antagonist and [B] is the concentration of the antagonist. Regression analysis of the plot log (concentration ratio − 1) against the log [B] (Schild plot) allowed us to confirm the competitive nature of the antagonist by assessing its slope (Arunlakshana and Schild, 1959).

In Vivo Functional Inhibitory Potency

Male Wistar rats (340–360 g) were anesthetized with sodium hexobarbital (150 mg/kg i.p.). After tracheal intubation, the rats were pithed with a steel rod and artificially ventilated with room air using a rodent ventilator (model 683, Harvard Apparatus, Southnatick, MA) at a tidal volume of 2 ml and a rate of 65 strokes/min. The animals were kept warm at 38°C. The femoral artery and vein were cannulated for blood pressure measurements and i.v. injection of drugs, respectively. After stabilization of blood pressure, various doses of bosentan or its vehicle (1 ml/kg) were injected. Five minutes later, the first dose of ET-1, big ET-1 or sarafotoxin S6C was injected i.v. in a volume of 0.5 ml/kg. Increasing doses were injected in a cumulative manner, with each dose being given after stabilization of the effect of the previous dose on blood pressure. In another set of experiments, the oral activity of bosentan was tested. Bosentan or its vehicle (5 ml/kg) was administered by gastric gavage with a cannula. Forty-five minutes later, the rat was anesthetized with sodium hexobarbital, pithed and ventilated. A single i.v. dose of big ET-1 was injected 60 to 65 min after the oral administration of bosentan. Finally, in a last set of experiments, the duration of action of orally given bosentan was studied by altering the time between the administration and the big ET-1 injection.

Expression of Results

The results are expressed as mean ± S.E.M. The effect of bosentan on a single dose of big ET-1 was assessed by using a variance analysis and a Dunnett's *t* test. Analysis of variance for univariate repeated measures was used to assess the effect of bosentan on dose-response curves of the different agonists *in vivo*. A P level less than .05 was considered significant.

Drugs

[^{125}I]-labeled ET-1 and [^{125}I-His]-labeled sarafotoxin S6C were obtained from Anawa Trading (Wangen, Switzerland). The [^{125}I]-labeled BQ-3020 was from Amersham International (Zürich, Switzerland). ET-1, big ET-1 and sarafotoxin S6C were obtained from Peninsula Laboratories (Merseyside, UK). They were dissolved in methanol:water (50:50) for *in vitro* studies or saline with 0.1% bovine serum albumin for *in vivo* studies. Dilutions were always performed in solutions that contained 0.1% bovine serum albumin. Bosentan was synthesized at Hoffmann-La Roche. For *in vitro* studies, bosentan (sodium salt or free sulfonamide) was dissolved in dimethyl sulfoxide. For oral administra-

tion, bosentan (micronized free sulfonamide) was used in suspension in 5% gum arabic and prepared freshly every day. For i.v. injection, bosentan (sodium salt) was dissolved in water immediately before use. Norepinephrine hydrochloride and potassium chloride were from Fluka Chemical (Buchs, Switzerland); acetylcholine hydrochloride, methoxamine and prostaglandin $F_{2\alpha}$ were obtained from Sigma (St. Louis, MO). Culture reagents, unless otherwise stated, were from Gibco Laboratories (Paisley, Scotland).

Results

Binding assays and selectivity. Bosentan competed with equal potency for binding of [^{125}I]-labeled ET-1 to ET_A receptor on human smooth muscle cells and rat mesangial cells (table 1). Similar K_i values were obtained for recombinant ET_A receptor expressed in CHO cells. However, with the membranes of baculovirus-infected insect cells that expressed the same receptor, an approximately 10-fold higher K_i value was observed.

On ET_B receptor-containing tissue, *i.e.*, membranes of human placenta in which endothelium represents a major structural component and which contain more than 90% ET_B receptors (data not shown) and of porcine cerebellum, bosentan displayed a 20- to 30-fold lower potency than on ET_A receptor-carrying cells. With recombinant ET_B receptors cloned from human placenta and expressed either on baculovirus-infected insect cells or CHO cells, K_i was 3- to 7-fold higher than on natural ET_B receptor-containing tissues (table 1).

Membranes from porcine trachea, which contracts in response to sarafotoxin S6C, were chosen to test the binding of bosentan to ET_{B2} receptors. Using [^{125}I]-labeled ET-1 as a ligand, microsomal membranes of porcine trachea displayed a high density of ET receptors (1.45 ± 0.07 pmol/mg of protein, $n = 5$). In the presence of the selective ET_A ligand BQ-123 (10 μM), the measured receptor density dropped to 83 ± 2 fmol/mg of protein ($n = 5$). Thus, about 6% of the ET receptors on tracheal membranes are of the ET_B receptor subtype. This receptor density was confirmed when the ET_B selective ligands [^{125}I]-labeled BQ-3020 and [^{125}I]-labeled sarafotoxin S6C were used to label the ET_B receptors specifically in tracheal membranes. B sentan competed with [^{125}I]-labeled BQ-3020 and [^{125}I]-labeled sarafotoxin S6C with similar K_i values (table 1). Direct-fit analysis of the competition binding curves with [^{125}I]-labeled BQ-3020 and sarafotoxin S6C on porcine trachea and with [^{125}I]-labeled ET-1 on the other ET_A or ET_B receptor-containing tissues using the LIGAND program revealed a single population of binding sites for bosentan. This was confirmed by the calculated Hill coefficients, which were in all cases close to unity (table 1), an observation which also excludes the involvement of cooperative binding phenomena.

To assess whether bosentan is a competitive antagonist of ET receptors, we performed saturation binding experiments on baculovirus-infected insect cells that expressed ET_A receptors and on membranes of human placenta by using [^{125}I]-labeled ET-1 as the tracer and varying concentrations of bosentan. On recombinant ET_A receptors (fig. 2A), we obtained apparent K_D values of 67 pM (control), 96 pM (with 50 nM bosentan) and 202 pM (with 100 nM bosentan). However, the apparent number of binding sites remained constant at 2967 ± 151 fmol/mg of protein. On human placental membranes (fig. 2B), we measured apparent K_D values of 15 pM (control), 26 pM (with 117 nM bosentan) and 71 pM (with 466 nM bosentan). Again, the apparent number of binding sites remained constant at 112 ± 3 fmol/mg of protein. These results are in agreement with a competitive binding mode of bosentan on both ET_A and ET_B receptors.

For assessing its selectivity, the binding inhibitory activity of bosentan against various peptides, eicosanoids and ions was tested. Up to a concentration of 10 μM, bosentan did not inhibit the binding of any of the ligands tested, except for the binding

TABLE 1
Binding potency of bosentan on different ET receptor subtypes

Receptor Subtype	Cell or Tissue	[^{125}I]-labeled Ligand	n	K_i of Bosentan	Hill Coefficient
ET_A	Recombinant (Sf9)	ET-1	7	43.0 ± 4.0	0.9 ± 0.1
	Recombinant (CHO)	ET-1	11	6.5 ± 0.4	0.9 ± 0.1
	HSMC[a]	ET-1	4	4.7 ± 0.4	0.8 ± 0.2
	RMC[b]	ET-1	5	4.1 ± 0.6	0.9 ± 0.1
ET_{B1}	Recombinant (Sf9)	ET-1	6	730 ± 73	0.9 ± 0.2
	Recombinant (CHO)	ET-1	3	343 ± 35	1.2 ± 0.2
	Human placenta	ET-1	5	95 ± 7	0.9 ± 0.1
	Porcine cerebellum	ET-1	3	152 ± 9	0.9 ± 0.1
ET_{B2}	Porcine trachea	BQ-3020	4	38 ± 6	1.2 ± 0.3
		SRTX S6C[c]	4	69 ± 12	1.5 ± 0.3

[a] Human smooth muscle cells.
[b] Rat mesangial cells.
[c] Sarafotoxin S6C.

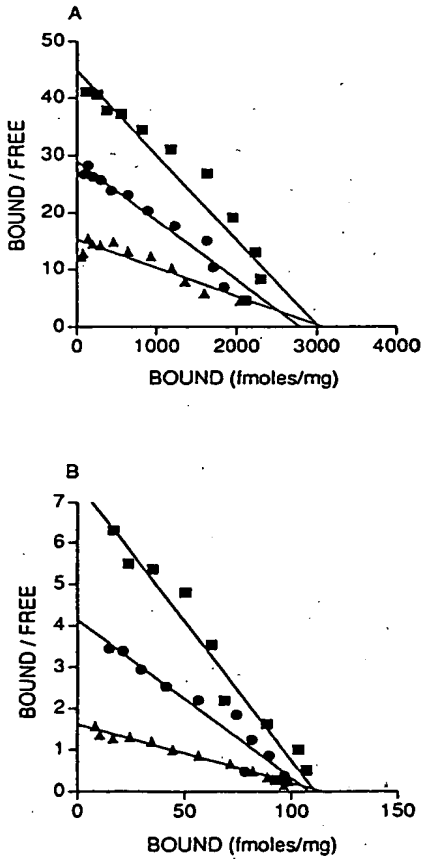


Fig. 2. Scatchard plots derived from saturation binding experiments. The values are the means of triplicate determinations from a representative experiment. A) Recombinant ET_A receptor; control (■) and in presence of 50 nM ( ) and 100 nM (▲) bosentan. B) Human placenta (ET_B); control (■) and in presence of 117 nM ( ) and 466 nM (▲) bosentan.

of neurokinin A, which was inhibited by 56.1% by 10 μM bosentan. This shows the high specificity of bosentan because the concentration required to inhibit the binding of ET-1 on native ET_A receptors by 56% was in the range of 1 nM.

***In vitro* functional studies.** Bosentan exhibited no agonist activity on any of the isolated tissue preparations studied. In isolated rat aortic rings denuded of their endothelium, ET-1 induced dose-dependent contraction, which was mediated through ET_A receptors. In preconstricted intact rabbit superior mesenteric artery, sarafotoxin S6C induced dose-dependent relaxation, which was mediated through endothelial ET_B (ET_{B1}) receptors. In rat tracheal rings denuded of epithelium, sarafotoxin S6C induced dose-dependent contraction, which was mediated through smooth muscle ET_B (ET_{B2}) receptors. In all three preparations, bosentan induced a parallel shift to the right in the concentration-response curves of ET-1 and sarafotoxin S6C (fig. 3), without any significant change in the maximal responses. In all three systems, Schild analysis yielded a slope not significantly different from unity, which suggested that bosentan also behaves in functional studies as a competitive antagonist on all three ET receptors. In rat aortic rings (ET_A receptors), Schild analysis indicated a pA_2 of 7.28 ± 0.04 (slope = 1.06 ± 0.12, n = 11). In rabbit superior mesenteric arteries (ET_{B1} receptors), the pA_2 was 6.72 ± 0.09 (slope = 0.88 ± 0.20, n = 11). In rat tracheal rings (ET_{B2} receptors), the pA_2 was 5.94 ± 0.04 (slope = 0.90 ± 0.18, n = 28).

Bosentan was also able to reverse an established contraction of isolated rat aortic rings to ET-1. After 1 hr of incubation with 500 pM ET-1, the rings contracted to a stable tension in the absence of antagonist, which could be relaxed concentration dependently by bosentan (fig. 4). The relaxation was slow, with a maximal effect at about 45 min. Half-maximal relaxation was achieved with a concentration of bosentan of 1.2×10^{-7} M.

***In vivo* functional studies.** Bosentan was itself devoid of effect on blood pressure. However, both after oral and i.v. administration, it significantly inhibited the pressor effect of big ET-1 (figs. 5 and 6). In contrast to big ET-1, which elicits only a pressor response, except at doses higher than 1 nmol/kg, i.v. ET induces an early decrease and a later and prolonged increase in blood pressure. Bosentan significantly inhibited both the depressor and the pressor response to ET-1 (fig. 7). Because of this dual inhibition of two opposite effects, the doses required to inhibit the pressor effect of ET-1 were higher than those needed to inhibit the effect of big ET-1. Bosentan also inhibited the depressor and pressor effects of the ET_B agonist sarafotoxin S6C (fig. 8). A dose of 10 mg/kg of i.v. bosentan was sufficient to inhibit completely the depressor component; only a dose of 30 mg/kg i.v. had a significant effect on the pressor response to sarafotoxin S6C.

The duration of action of bosentan was tested by measuring the inhibition of action of big ET-1 at different times after administration. At a dose of 30 mg/kg orally, it inhibited the effect of 0.3 nmol/kg of big ET-1 injected 45 min and 6 hr later by 61% ($P < .01$, n = 3) and 65% ($P < .01$, n = 3), respectively. At 24 hr, the inhibition was 16% (n = 4). At a dose of 100 mg/kg orally, bosentan still inhibited the effect of 0.3 nmol/kg of big ET-1 by 37% 24 hr after its administration ($P < .05$, n = 4).

Discussion

Because ET is suggested to play a role in chronic diseases, such as hypertension, congestive heart failure or arteriosclerosis (Lüscher *et al.*, 1993; McMurray *et al.*, 1992; Lerman *et al.*, 1991), the development of ET receptor antagonists with oral activity is essential. We recently described Ro 46–2005 as the first orally active ET receptor antagonist (Clozel *et al.*, 1993a). Like Ro 46–2005, bosentan is a mixed and competitive antagonist of ET receptors. However, it has a different profile, being more potent and more selective for ET_A receptors than Ro 46–2005 is.

The binding inhibitory potency of bosentan was tested on recombinant human receptors (for ET_A and ET_{B1} receptors) and on natural receptors of human or animal sources. No fundamental difference in its binding was apparent between human and animal receptors. Therefore, it seemed justified to use animal tissues for evaluating the functional inhibitory

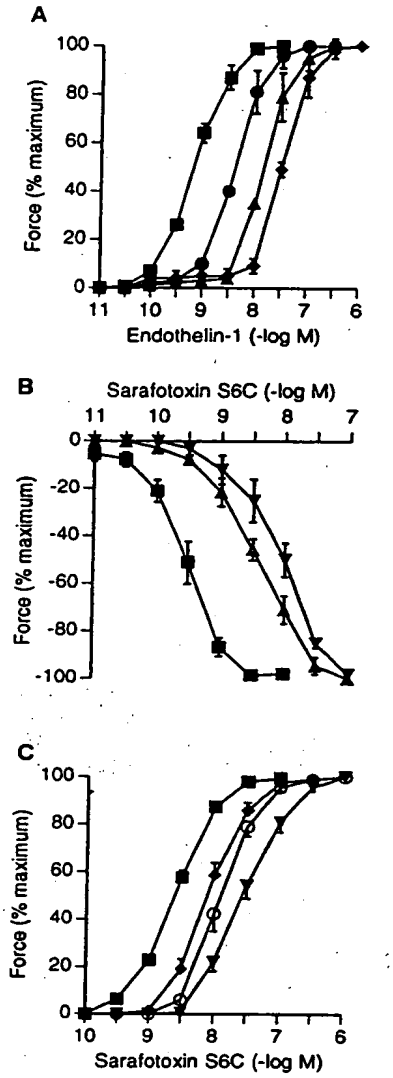


Fig. 3. Effect of bosentan (-●-, 0.3 μM; -▲-, 1 μM; -◆-, 3 μM, -○-, 7 μM and -▼-, 10 μM) or its vehicle (DMSO, -■-) on the concentration-response curves of A) ET-1-mediated contraction of isolated rat aorta without endothelium, B) sarafotoxin S6C-mediated endothelium-dependent relaxation of precontracted rabbit superior mesenteric artery, C) sarafotoxin S6C-mediated contraction in isolated rat trachea without epithelium.

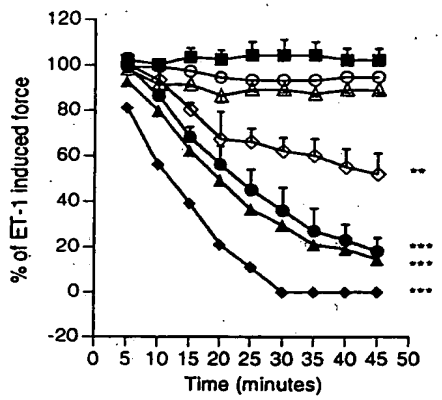


Fig. 4. Relaxation by bosentan of the contraction induced by ET-1 (500 pM) on rat aortic rings without endothelium. Bosentan (-○-, 10 nM; -△-, 30 nM; -◇-, 100 nM; -●-, 300 nM; -▲-, 1 μM and -◆-, 3 μM) or its vehicle (water, -■-) was added 60 min after ET-1. The values are the means ± S.E.M. of three experiments. **P < .01, ***P < .001 compared with vehicle.

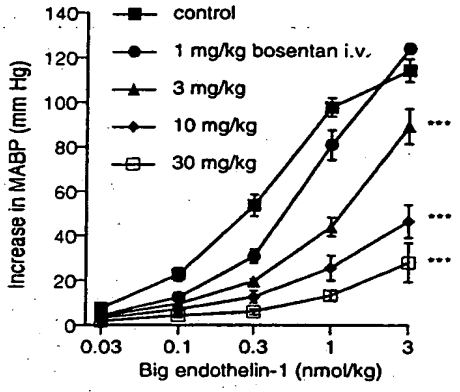


Fig. 5. Effect of i.v. administered bosentan (1–30 mg/kg, n = 4–5) or salin (n = 13) on the increase in mean arterial blood pressure (MABP) induced by increasing doses of i.v. big ET-1 in pithed rats. ***P < .001 compared with control.

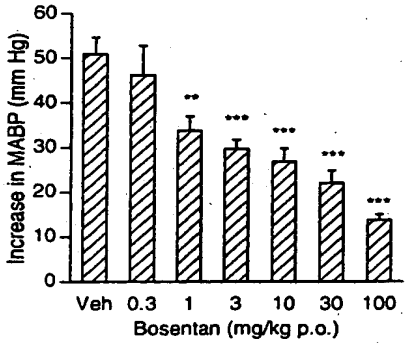
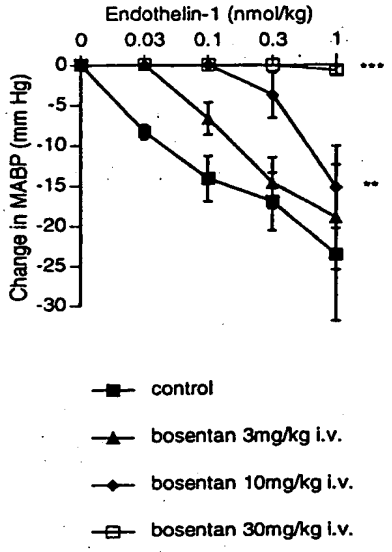


Fig. 6. Effect of orally administered bosentan (n = 4–8) or its vehicle (veh, n = 10) on the increase in MABP induced by i.v. big ET-1 (0.3 nmol/kg) in pithed rats. **P < .01, ***P < .001 compared with vehicle.

effects of bosentan. Further studies are mandatory for assessing the effects of bosentan on human tissues because the relative distribution of ET receptors may vary between species and bosentan does not have similar affinities for all three receptors.



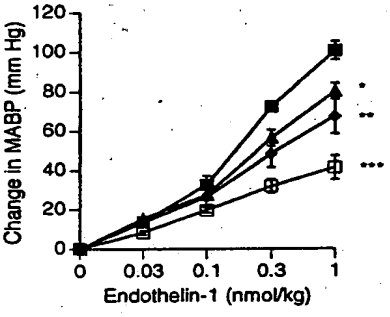


Fig. 7. Inhibition of the depressor and pressor responses to increasing doses of i.v. ET-1 by i.v. bosentan (n = 3–4) in pithed rats. *P < .05, **P < .01, ***P < .001 compared with control.

The affinity of bosentan was similar in the different tests used for each receptor subtype. However, on the ET receptors expressed on the Sf9 cells, the K_i values were 2- to 7-fold higher than on other systems. This could either reflect an incomplete glycosylation of receptors that were expressed on baculovirus-infected insect cells (Kuroda *et al.*, 1990) or a different coupling to G-proteins, which would lead to conformational changes of the receptor. Such conformational changes could also be induced by the high receptor density on the membranes of Sf9 cells. As an example, the receptor density per milligram of protein calculated by the LIGAND program was higher for the recombinant ET_A (3.8 ± 0.3 pmol/mg) or ET_{B1} (23 ± 5.5 pmol/mg) receptor expressed on baculovirus-infected Sf9 cells compared with natural systems, such as membranes of human placenta (0.46 ± 0.04 pmol/mg) or porcine cerebellum (0.46 ± 0.03 pmol/mg). Overall, both in binding and in functional experiments, bosentan was most potent on ET_A receptors. Its binding affinity for ET_{B1} and ET_{B2} receptors was 35- and 11-fold lower, respectively, than for the ET_A receptor but the order of potency was inverted for the two ET_B receptors in functional studies. This different order of potency between binding and functional studies is probably explained by the different test systems and experimental conditions. On rat aortic rings, bos-

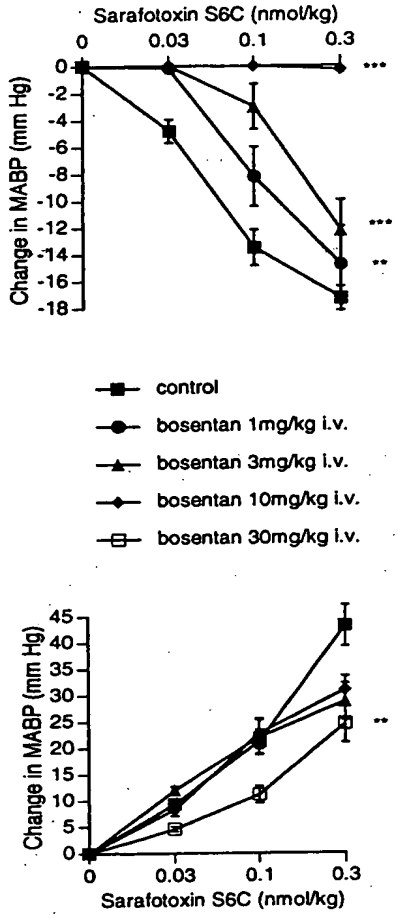


Fig. 8. Inhibition of the depressor and pressor responses to increasing doses of i.v. sarafotoxin S6C by i.v. bosentan (n = 4–7) in pithed rats. **P < .01, ***P < .001 compared with control.

entan was capable of reversing an established contraction to ET-1, with a half-maximal effect at a concentration (1.2×10^{-7} M), which was consistent with the apparent dissociation constant estimated by the pA_2 determination (7×10^{-8} M). This finding was in agreement with the data on BQ-123, which was shown to relax aortic rings precontracted by ET-1 (Marsault *et al.*, 1993). By preventing binding of new ET-1 molecules, these antagonists prevent maintenance of contraction to ET-1 because the sustained constricting effect mediated by ET_A receptors seems to be due to continuous binding of ET-1 molecules to recycling free receptors after internalization and intracellular deactivation (Marsault *et al.*, 1993).

In vivo, the major part of the pressor effect of big ET-1 or ET-1 is considered ET_A mediated but a small component is resistant to inhibition by a selective ET_A antagonist, BQ-123 (McMurdo *et al.*, 1993). In contrast, the pressor effect of sarafotoxin S6C, which is not due to secondary stimulation of ET_A receptors because BQ-123 is inactive (Clozel *et al.*, 1992), by definition, is mediated through activation of ET_B (ET_{B2}) receptors. Finally, the decrease in blood pressure observed with i.v. ET-1 or sarafotoxin S6C is unaffected or even increased by selective ET_A receptor antagonists (Ihara *et al.*, 1992; McMurdo *et al.*, 1993) and is due to the activation of endothelial ET_{B1} receptors and release of endothelium-derived relaxing factors. Our results showed that bosentan inhibited responses mediated through the *in vivo* activation of these three receptors. The highest *in vivo* potency of bosentan was the inhibition of the pressor response to big ET-1. Higher doses are needed to inhibit the pressor response to ET-1 because the inhibitory effect of bosentan is then moderated by the concomitant inhibition of its depressor effect. The inhibition of the depressor response to ET-1 is achieved with relatively low doses of bosentan compared with the doses needed to block pressor responses, probably because it reaches effective concentrations in the endothelial layer more easily than in the media. In contrast to ET-1, i.v. big ET-1 does not induce a depressor effect (Clozel *et al.*, 1993b). Thus, a mixed receptor antagonist, such as bosentan, can inhibit the action of a pure pressor agonist (such as big ET-1) with lower doses than those needed to inhibit the pressor effect of an agonist that induces a dual effect (such as ET-1). Using a mixed antagonist that is less potent than bosentan on ET_A receptors, Ro 46–2005, we even described a potentiation of the pressor effect of ET-1 at doses of antagonist that markedly inhibited the pressor effect of big ET-1 (Clozel *et al.*, 1993b). Higher doses were necessary to inhibit both the pressor and depressor effects of ET-1. Our hypothesis is that the fate of big ET-1, which needs to be converted in the tissues into mature ET-1 before acting, reproduces the natural route of ET-1 release better than does the i.v. injection of ET-1 and that the depressor effect of i.v. injected ET-1 might be a pharmacological observation (Clozel *et al.*, 1993b). To ascertain that the greater inhibitory potency of bosentan on big ET-1 than ET-1 could not be explained by an effect of bosentan on the conversion of big ET-1 to ET-1, we tested the effect of bosentan on ET-converting enzyme activity in membranes prepared from a human endothelial cell line. Bosentan at concentrations up to 10^{-3} M was devoid of any inhibitory effect on ET-converting enzyme activity. Finally, in addition to the ET_A- and ET_{B1}-mediated effects, the ET_{B2}-mediated responses (pressor effect of sarafotoxin S6C) were inhibited with the highest doses of bosentan. Overall, these results confirm the inhibitory potency of bosentan on the different ET receptors. The observation that bosentan virtually abolished the pressor effect of low doses of big ET-1 suggests that all receptors involved in the pressor response to ET-1 were blocked by the antagonist. After a single oral administration at 100 mg/kg, bosentan was still effective after 1 day in the inhibition of the pressor effect of 0.3 nmol/kg of big ET-1, which showed that, at this dose, the compound has a 24-hr duration of action.

Overall, its selectivity, its inhibition of all known ET receptors, its oral activity and its duration of action make bosentan a useful tool to evaluate the chronic effects of ET receptor antagonism. In conjunction with ET_A-selective antagonists, it may be used especially to evaluate the contribution of the novel "constricting" ET_B receptor and of the ET_A receptor in the pathophysiology of vasospasm in animal models. Finally, it may represent a new therapeutic tool in the management of acute and chronic vasoconstriction.

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